



**PCT**

**pMAK705deltaaspA**

7141 bps

cat

rep-ts

6000

5000

4000

3000

2000

1000

7000

aspA5'

aspA3'

EcoRI, 870

EcoRI, 2281

XbaI, 2308

EcoRI, 2353

EcoRI, 3083

EcoRI, 3130

HindIII, 3822

HindIII, 3871

[illegible]

WC 33/008603 A2



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*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**Process for the Preparation of L-Amino Acids using Strains  
of the Enterobacteriaceae Family which Contain an  
Attenuated aspA Gene**

**Field of the Invention**

- 5 This invention relates to a process for the fermentative preparation of L-amino acids, in particular L-threonine, using strains of the Enterobacteriaceae family in which the aspA gene is attenuated.

**Prior Art**

- 10 L-Amino acids, in particular L-threonine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

- It is known to prepare L-amino acids by fermentation of  
15 strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as e.g.  
20 stirring and supply of oxygen, or the composition of the nutrient media, such as e.g. the sugar concentration during the fermentation, or the working up to the product form, by e.g. ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

- 25 Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the threonine analogue  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid (AHV), or are auxotrophic for  
30 metabolites of regulatory importance and produce L-amino acid, such as e.g. L-threonine, are obtained in this

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the production.

#### Object of the Invention

The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in particular L-threonine.

#### Summary of the Invention

The invention provides a process for the fermentative preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which in particular already produce L-amino acids and in which the nucleotide sequence which codes for the aspA gene is attenuated.

#### Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the

corresponding enzyme (protein) or gene, and optionally combining these measures.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

The process comprises carrying out the following steps:

- 10 a) fermentation of microorganisms of the Enterobacteriaceae family in which the aspA gene is attenuated,
  - b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and
  - 15 c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.
- 20 The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen
- 25 from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. Of the genus Escherichia the species Escherichia coli and of the genus Serratia the species Serratia marcescens are to be mentioned in particular.
- 30 Suitable strains, which produce L-threonine in particular, of the genus Escherichia, in particular of the species Escherichia coli, are, for example,

- Escherichia coli TF427
- Escherichia coli H4578
- Escherichia coli KY10935
- Escherichia coli VNIIGenetika MG442
- 5 Escherichia coli VNIIGenetika M1
- Escherichia coli VNIIGenetika 472T23
- Escherichia coli BKIIM B-3996
- Escherichia coli kat 13
- Escherichia coli KCCM-10132

- 10 Suitable L-threonine-producing strains of the genus *Serratia*, in particular of the species *Serratia marcescens*, are, for example

- Serratia marcescens* HNr21
- Serratia marcescens* TLr156
- 15 *Serratia marcescens* T2000

- Strains from the Enterobacteriaceae family which produce L-threonine preferably have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of:
- 20 resistance to  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to  $\alpha$ -methylserine, resistance to diaminosuccinic acid, resistance to  $\alpha$ -aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate,
  - 25 resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a partial and compensable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine,
  - 30 resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid, resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-methionine,
  - 35 sensitivity to fluoropyruvate, defective threonine

dehydrogenase, optionally an ability for sucrose utilization, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feed back resistant form, enhancement  
5 of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feed back resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feed back resistant form,  
10 enhancement of phosphoenol pyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.

15 It has been found that microorganisms of the Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner after attenuation, in particular elimination, of the aspA gene.

The nucleotide sequences of the genes of Escherichia coli  
20 belong to the prior art and can also be found in the genome sequence of Escherichia coli published by Blattner et al. (Science 277: 1453 - 1462 (1997)).

The aspA gene is described, inter alia, by the following data:

25 Description: Aspartate ammonium lyase (aspartase)  
EC No.: 4.3.1.1  
Reference: Takagi et al.; Nucleic Acids Research  
13(6): 2063-2074 (1985); Woods et al.;  
Biochemical Journal 237(2): 547-557 (1986);  
30 Falzone et al.; Biochemistry 27(26): 9089-  
9093 (1988); Jayasekera et al.;  
Biochemistry 36(30): 9145-9150 (1997)  
Accession No.: AF000486

The nucleic acid sequences can be found in the databanks of the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (Bethesda, MD, USA), the nucleotide sequence databank of the European Molecular  
5 Biologies Laboratories (EMBL, Heidelberg, Germany or Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima, Japan).

The genes described in the text references mentioned can be used according to the invention. Alleles of the genes which  
10 result from the degeneracy of the genetic code or due to "sense mutations" of neutral function can furthermore be used.

To achieve an attenuation, for example, expression of the gene or the catalytic properties of the enzyme proteins can  
15 be reduced or eliminated. The two measures can optionally be combined.

The reduction in gene expression can take place by suitable culturing, by genetic modification (mutation) of the signal structures of gene expression or also by the antisense-RNA  
20 technique. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The expert can find information in this respect, inter alia, for example, in Jensen and Hammer  
25 (Biotechnology and Bioengineering 58: 191-195 (1998)), in Carrier and Keasling (Biotechnology Progress 15: 58-64 (1999)), Franch and Gerdes (Current Opinion in Microbiology 3: 159-164 (2000)) and in known textbooks of genetics and molecular biology, such as, for example, the textbook of  
30 Knippers ("Molekulare Genetik [Molecular Genetics]", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that of Winnacker ("Gene und Klone [Genes and Clones]", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).



Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art. Examples which may be mentioned are the works of Qiu and Goodman (Journal of Biological Chemistry 272: 8611-  
5 8617 (1997)), Yano et al. (Proceedings of the National Academy of Sciences, USA 95: 5511-5515 (1998)), Wentz and Schachmann (Journal of Biological Chemistry 266: 20833-20839 (1991)). Summarizing descriptions can be found in known textbooks of genetics and molecular biology, such as  
10 e.g. that by Hagemann ("Allgemeine Genetik [General Genetics]", Gustav Fischer Verlag, Stuttgart, 1986).

Possible mutations are transitions, transversions, insertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, "missense  
15 mutations" or "nonsense mutations" are referred to. Insertions or deletions of at least one base pair in a gene lead to "frame shift mutations", which lead to incorrect amino acids being incorporated or translation being interrupted prematurely. If a stop codon is formed in the  
20 coding region as a consequence of the mutation, this also leads to a premature termination of the translation. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on generation of such mutations are prior art and can be found in known  
25 textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik [Molecular Genetics]", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone [Genes and Clones]", VCH Verlagsgesellschaft, Weinheim, Germany,  
30 1990) or that by Hagemann ("Allgemeine Genetik [General Genetics]", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations in the genes, such as, for example, deletion mutations, can be incorporated into suitable strains by gene or allele replacement.

A conventional method is the method, described by Hamilton et al. (Journal of Bacteriology 171: 4617-4622 (1989)), of gene replacement with the aid of a conditionally replicating pSC101 derivative pMAK705. Other methods described in the prior art, such as, for example, those of Martinez-Morales et al. (Journal of Bacteriology 181: 1999, 7143-7148 (1999)) or those of Boyd et al. (Journal of Bacteriology 182: 842-847 (2000)), can likewise be used.

It is also possible to transfer mutations in the particular genes or mutations which affect expression of the particular genes into various strains by conjugation or transduction.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family, in addition to attenuation of the aspA gene, for one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate or enzymes of glycolysis or PTS enzymes or enzymes of sulfur metabolism to be enhanced.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene which codes for a corresponding enzyme or protein with a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or more, based on that of the wild-type protein or the

activity or concentration of the protein in the starting microorganism.

Thus, for example, at the same time one or more of the genes chosen from the group consisting of

- 5 • the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the pyc gene of *Corynebacterium glutamicum* which codes for pyruvate carboxylase (WO 99/18228),
- 10 • the pps gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231(2): 332-336 (1992)),
- the ppc gene which codes for phosphoenol pyruvate carboxylase (Gene 31: 279-283 (1984)),
- 15 • the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- the rhtB gene which imparts homoserine resistance (EP-A-0 994 190),
- the mqo gene which codes for malate:quinone  
20 oxidoreductase (WO 02/06459),
- the rhtC gene which imparts threonine resistance (EP-A-1 013 765),
- the thrE gene of *Corynebacterium glutamicum* which codes for the threonine export protein (WO 01/92545),
- 25 • the gdhA gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983)),

- the hns gene which codes for the DNA-binding protein HLP-II (Molecular and General Genetics 212: 199-202 (1988)),
- 5 • the pgm gene which codes for phosphoglucomutase (Journal of Bacteriology 176: 5847-5851 (1994)),
- the fba gene which codes for fructose biphosphate aldolase (Biochemical Journal 257: 529-534 (1989)),
- 10 • the ptsH gene of the ptsHIcrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- the ptsI gene of the ptsHIcrr operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- 15 • the crr gene of the ptsHIcrr operon which codes for the glucose-specific IIA component of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- 20 • the ptsG gene which codes for the glucose-specific IIBC component (Journal of Biological Chemistry 261: 16398-16403 (1986)),
- the lrp gene which codes for the regulator of the leucine regulon (Journal of Biological Chemistry 266: 10768-10774 (1991)),
- 25 • the mopB gene which codes for 10 Kd chaperone (Journal of Biological Chemistry 261: 12414-12419 (1986)) and is also known by the name groES,
- the ahpC gene of the ahpCF operon which codes for the small sub-unit of alkyl hydroperoxide reductase  
30 (Proceedings of the National Academy of Sciences of the United States of America 92: 7617-7621 (1995)),

- the *ahpF* gene of the *ahpCF* operon which codes for the large sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92: 7617-7621 (1995)),
- 5 • the *cysK* gene which codes for cysteine synthase A (Journal of Bacteriology 170: 3150-3157 (1988)),
- the *cysB* gene which codes for the regulator of the *cys* regulon (Journal of Biological Chemistry 262: 5999-6005 (1987)),
- 10 • the *cysJ* gene of the *cysJIH* operon which codes for the flavoprotein of NADPH sulfite reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
- the *cysI* gene of the *cysJIH* operon which codes for the  
15 haemoprotein of NADPH sulfite reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)) and
- the *cysH* gene of the *cysJIH* operon which codes for  
20 adenyl sulfite reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989))

can be enhanced, in particular over-expressed.

The use of endogenous genes is in general preferred.

"Endogenous genes" or "endogenous nucleotide sequences" are  
25 understood as meaning the genes or nucleotide sequences present in the population of a species.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to attenuation of the *aspA* gene, for one or more of the genes  
30 chosen from the group consisting of

- the tdh gene which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- 5 • the gene product of the open reading frame (orf) yjfA (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 of the National Center for  
10 Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the pckA gene which codes for the enzyme phosphoenol pyruvate carboxykinase (Journal of Bacteriology 172: 7151-7156 (1990)),
- the poxB gene which codes for pyruvate oxidase (Nucleic  
15 Acids Research 14(13): 5449-5460 (1986)),
- the aceA gene which codes for the enzyme isocitrate lyase (Journal of Bacteriology 170: 4528-4536 (1988)),
- the dgsA gene which codes for the DgsA regulator of the phosphotransferase system (Bioscience, Biotechnology and  
20 Biochemistry 59: 256-261 (1995)) and is also known under the name of the mlc gene,
- the fruR gene which codes for the fructose repressor (Molecular and General Genetics 226: 332-336 (1991)) and is also known under the name of the cra gene,
- 25 • the rpoS gene which codes for the sigma<sup>38</sup> factor (WO 01/05939) and is also known under the name of the katF gene,
- the aceB gene which codes for malate synthase A (Nucleic Acids Research 14(13): 5449-5460 (1986)),

- the aceK gene which codes for isocitrate dehydrogenase kinase/phosphatase (Journal of Bacteriology 170(1): 89-97 (1988)) and
- the ugpB gene which codes for the periplasmic binding protein of the sn-glycerol 3-phosphate transport system (Molecular Microbiology 2(6): 767-775 (1988))

to be attenuated, in particular eliminated or for the expression thereof to be reduced.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to attenuation of the aspA gene, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed batch process (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as  
5 e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

- 10 Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be  
15 used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus.

- 20 The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances.  
25 Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

- Basic compounds, such as sodium hydroxide, potassium  
30 hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture.

Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Other substances having a selective action, e.g. antibiotics, can

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be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the  
5 culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino acids or L-threonine has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion  
10 exchange chromatography with subsequent ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958)), or it can take place by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

15 The process according to the invention is used for the fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

A pure culture of the Escherichia coli K-12 strain  
20 DH5 $\alpha$ /pMAK705 was deposited as DSM 13720 on 8th September 2000 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

25 The present invention is explained in more detail in the following with the aid of embodiment examples.

The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, ligation, Klenow and alkaline phosphatase treatment are carried out by the method of  
30 Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). Unless described otherwise, the transformation of Escherichia coli is carried out by the method of Chang et al. (Yeast 1988)

of the National Academy of Sciences of the United States of America 86: 2172-2175 (1989)).

The incubation temperature for the preparation of strains and transformants is 37°C. Temperatures of 30°C and 44°C  
5 are used in the gene replacement method of Hamilton et al.

#### Example 1

Construction of the deletion mutation of the aspA gene

Parts of the gene regions lying upstream and downstream of the aspA gene and parts of the 5' and 3' region of the aspA  
10 gene are amplified from Escherichia coli K12 using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the aspA gene and sequences lying upstream and downstream in E. coli K12 MG1655 (SEQ ID No. 1, Accession Number  
15 AE000486 and AE000487), the following PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

aspA5'-1: 5' - GCTGCATCAGCACGAAATTC - 3' (SEQ ID No. 3)

aspA5'-2: 5' - CCATTACCATACCGCGAACA - 3' (SEQ ID No. 4)

aspA3'-1: 5' - TGGCAGCAGAAGCAGGTCAG - 3' (SEQ ID No. 5)

20 aspA3'-2: 5' - TAGTCCAGACCGCCAGCAAC - 3' (SEQ ID No. 6)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 650 bp in size from the 5' region of  
25 the aspA gene region (called aspA5') and a DNA fragment approx. 700 bp in size from the 3' region of the aspA gene region (called aspA3') can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications,  
30 Academic Press) with Taq-DNA polymerase (Gibco-BRL, Eggenstein, Germany). The PCR products are each ligated

with the vector pCR2.1-TOPO (TOPO TA Cloning Kit, Invitrogen, Groningen, The Netherlands) in accordance with the manufacturer's instructions and transformed into the E. coli strain TOP10F'. Selection of plasmid-carrying cells  
5 takes place on LB agar, to which 50 µg/ml ampicillin are added. After isolation of the plasmid DNA, the vector pCR2.1-TOPOaspA3' is cleaved with the restriction enzymes XbaI and Ecl136II. The aspA3' fragment is isolated after separation in 0.8% agarose gel with the aid of the QIAquick  
10 Gel Extraction Kit (QIAGEN, Hilden, Germany). After isolation of the plasmid DNA the vector pCR2.1-TOPOaspA5' is cleaved with the enzymes EcoRV and XbaI and ligated with the aspA3' fragment isolated. The E. coli strain DH5α is transformed with the ligation batch and plasmid-carrying  
15 cells are selected on LB agar, to which 50 µg/ml ampicillin are added. After isolation of the plasmid DNA those plasmid in which the mutagenic DNA sequence shown in SEQ ID No. 7 is cloned are detected by control cleavage with the enzymes EcoRI, XbaI and HindIII. One of the plasmids is called  
20 pCR2.1-TOPOΔaspA (=pCR2.1-TOPOdeltaaspA).

### Example 2

#### Construction of the replacement vector pMAK705ΔaspA

The ΔaspA allele described in example 1 is isolated from the vector pCR2.1-TOPOΔaspA after restriction with the  
25 enzymes HindIII and XbaI and separation in 0.8% agarose gel, and ligated with the plasmid pMAK705 (Hamilton et al., Journal of Bacteriology 171: 4617-4622 (1989)), which has been digested with the enzymes HindIII and XbaI. The ligation batch is transformed in DH5α and plasmid-carrying  
30 cells are selected on LB agar, to which 20 µg/ml chloramphenicol are added. Successful cloning is demonstrated after isolation of the plasmid DNA and cleavage with the enzymes HindIII and XbaI. The replacement vector formed, pMAK705ΔaspA (= pMAK705deltaaspA), is shown  
35 in Figure 1.

Example 3

Position-specific mutagenesis of the aspA gene in the E. coli strain MG442

The L-threonine-producing E. coli strain MG442 is described  
5 in the patent specification US-A- 4,278,765 and deposited  
as CMIM B-1628 at the Russian National Collection for  
Industrial Microorganisms (VKPM, Moscow, Russia).

For replacement of the chromosomal aspA gene with the  
plasmid-coded deletion construct, MG442 is transformed with  
10 the plasmid pMAK705 $\Delta$ aspA. The gene replacement is carried  
out using the selection method described by Hamilton et al.  
(Journal of Bacteriology 171: 4617-4622 (1989)) and is  
verified by standard PCR methods (Innis et al. (1990) PCR  
Protocols. A Guide to Methods and Applications, Academic  
15 Press) with the following oligonucleotide primers:

aspA5'-1: 5' - GCTGCATCAGCACGAAATTC - 3' (SEQ ID No. 3)

aspA3'-2: 5' - TAGTCCAGACCGCCAGCAAC - 3' (SEQ ID No. 6)

After replacement has taken place, MG442 contains the form  
of the  $\Delta$ aspA allele shown in SEQ ID No. 8. The strain  
20 obtained is called MG442 $\Delta$ aspA.

Example 4

Preparation of L-threonine with the strain MG442 $\Delta$ aspA

MG442 $\Delta$ aspA is multiplied on minimal medium with the  
following composition: 3.5 g/l Na<sub>2</sub>HPO<sub>4</sub>\*2H<sub>2</sub>O, 1.5 g/l KH<sub>2</sub>PO<sub>4</sub>,  
25 1 g/l NH<sub>4</sub>Cl, 0.1 g/l MgSO<sub>4</sub>\*7H<sub>2</sub>O, 2 g/l glucose, 20 g/l agar.  
The formation of L-threonine is checked in batch cultures  
of 10 ml contained in 100 ml conical flasks. For this,  
10 ml of preculture medium of the following composition:  
2 g/l yeast extract, 10 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g/l KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/l  
30 MgSO<sub>4</sub>\*7H<sub>2</sub>O, 15 g/l CaCO<sub>3</sub>, 20 g/l glucose are inoculated and  
the batch is incubated for 16 hours at 37°C and 180 rpm or.

- an ESR incubator from Kühner AG (Birsfelden, Switzerland). 250 µl of this preculture are transinoculated into 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose) and the batch is incubated for 48 hours at 37°C. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.
- 10 The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.
- 15 The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	L-Threonine g/l
MG442	6.0	1.5
MG442 $\Delta$ aspA	5.5	1.9

## Brief Description of the Figure:

- Figure 1: pMAK705 $\Delta$ aspA ( = pMAK705 $\Delta$ aspA)
- 20 The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:
- cat: Chloramphenicol resistance gene
  - rep-ts: Temperature-sensitive replication region of the plasmid pSC101
- 25

- aspA5': Part of the 5' region of the aspA gene and the region lying upstream
  - aspA3': Part of the 3' region of the aspA gene and the region lying downstream
- 5 The abbreviations for the restriction enzymes have the following meaning
- EcoRI: Restriction endonuclease from Escherichia coli
  - HindIII: Restriction endonuclease from Haemophilus influenza
- 10 • XbaI: Restriction endonuclease from Xanthomonas badrii

**What is claimed is:**

1. A process for the preparation of L-amino acids, in particular L-threonine, which comprises carrying out the following steps:
  - 5 a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which the aspA gene, or the nucleotide sequence which codes for this, is attenuated, in particular eliminated,
  - 10 b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and
  - c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof
  - 15 optionally remaining in the product.
2. A process as claimed in claim 1, wherein microorganisms in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.
- 20 3. A process as claimed in claim 1, wherein microorganisms in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
4. A process as claimed in claim 1, wherein the expression
- 25 of the polynucleotide which codes for the aspA gene is attenuated, in particular eliminated.
5. A process as claimed in claim 1, wherein the regulatory and/or catalytic properties of the polypeptide (enzyme protein) for which the polynucleotide aspA codes are
- 30 reduced.

6. A process as claimed in claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:
- 5
- 6.1 the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
- 6.2 the pyc gene which codes for pyruvate carboxylase,
- 10
- 6.3 the pps gene which codes for phosphoenol pyruvate synthase,
- 6.4 the ppc gene which codes for phosphoenol pyruvate carboxylase,
- 15
- 6.5 the pntA and pntB genes which code for transhydrogenase,
- 6.6 the rhtB gene which imparts homoserine resistance,
- 6.7 the mgo gene which codes for malate:quinone oxidoreductase,
- 20
- 6.8 the rhtC gene which imparts threonine resistance,
- 6.9 the thrE gene which codes for the threonine export protein,
- 25
- 6.10 the gdhA gene which codes for glutamate dehydrogenase,
- 6.11 the hns gene which codes for the DNA-binding protein HLP-II.



- 6.12 the pgm gene which codes for phosphoglucomutase,
- 6.13 the fba gene which codes for fructose biphosphate aldolase,
- 5 6.14 the ptsH gene which codes for the phosphohistidine protein hexose phosphotransferase,
- 6.15 the ptsI gene which codes for enzyme I of the phosphotransferase system,
- 10 6.16 the crr gene which codes for the glucose-specific IIA component,
- 6.17 the ptsG gene which codes for the glucose-specific IIBC component,
- 15 6.18 the lrp gene which codes for the regulator of the leucine regulon,
- 6.19 the mopB gene which codes for 10 Kd chaperone,
- 6.20 the ahpC gene which codes for the small sub-unit of alkyl hydroperoxide reductase,
- 20 6.21 the ahpF gene which codes for the large sub-unit of alkyl hydroperoxide reductase,
- 6.22 the cysK gene which codes for cysteine synthase A,
- 6.23 the cysB gene which codes for the regulator of the cys regulon,
- 25 6.24 the cysJ gene which codes for the flavoprotein of NADPH sulfite reductase,
- 6.25 the cysI gene which codes for the haemoprotein of NADPH sulfite reductase and

6.26 the cysH gene which codes for adenylyl sulfate reductase,

is or are enhanced, in particular over-expressed, are fermented.

5 7. A process as claimed in claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:

- 10 7.1 the tdh gene which codes for threonine dehydrogenase,
- 7.2 the mdh gene which codes for malate dehydrogenase,
- 15 7.3 the gene product of the open reading frame (orf) yjfA,
- 7.4 the gene product of the open reading frame (orf) ytfP,
- 7.5 the pckA gene which codes for phosphoenolpyruvate carboxykinase
- 20 7.6 the poxB gene which codes for pyruvate oxidase
- 7.7 the aceA gene which codes for isocitrate lyase,
- 7.8 the dgsA gene which codes for the DgsA regulator of the phosphotransferase system,
- 25 7.9 the fruR gene which codes for the fructose repressor,
- 7.10 the rpoS gene which codes for the sigma<sup>38</sup> factor,
- 7.11 the tdk gene which codes for threonine synthase.

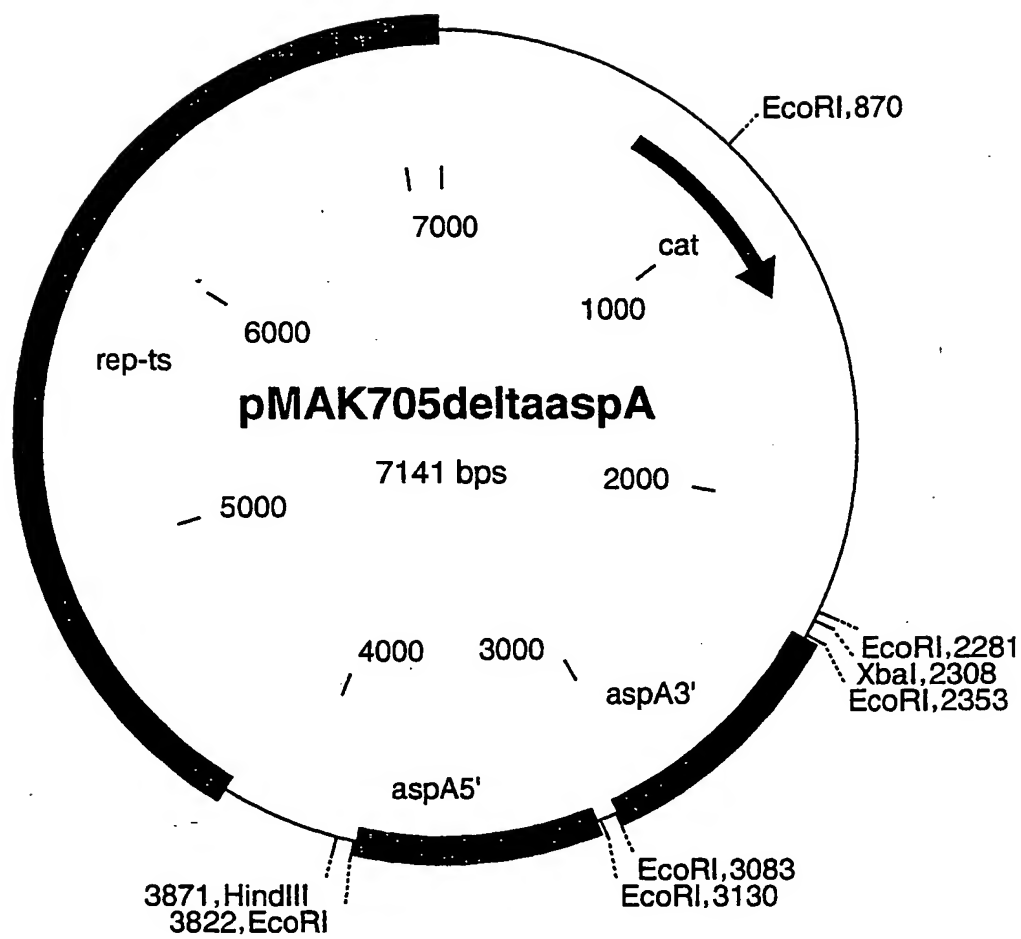
7.12 the aceK gene which codes for isocitrate dehydrogenase kinase/phosphatase and

7.13 the ugpB gene which codes for the periplasmic binding protein of the sn-glycerol 3-phosphate transport system

5

is or are attenuated, in particular eliminated or reduced in expression, are fermented.

Figure 1:



## SEQUENCE PROTOCOL

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tgatgcaccc ggcttacaaa gcaaaacgct atactgatga aagcgaacag taa 653

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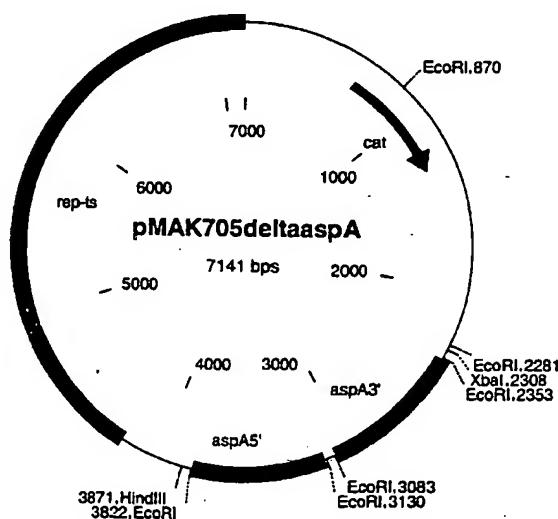
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— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

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(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEA FAMILY WHICH CONTAIN AN ATTENUATED ASPA GENE



(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which the aspA gene, or the nucleotide sequence which codes for this, is attenuated, in particular eliminated, b) concentration of the L-amino acid in the medium or in the cells of the bacteria, and c) isolation of the L-amino acid.

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International Application No

PCT/EP 02/07351

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12P13/08 C12P13/12 C12P13/14 C12P13/06 C12P13/10  
C12P13/20 C12P13/22 C12P13/24

According to International Patent Classification (IPC) or to both national classification and IPC

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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☒ Further documents are listed in the continuation of box C.

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International Application No

PCT/EP 02/07351

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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International Application No

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Information on patent family members

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